

Antihypertensive Mechanism of Lactoferrin-Derived Peptides: Angiotensin Receptor Blocking Effect

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ABSTRACT: Looking for antihypertensive mechanisms beyond ACE inhibition, we assessed whether lactoferrin (LF)-derived peptides can act as receptor blockers to inhibit vasoconstriction induced by angiotensin II or endothelin-1. The lactoferricinB (LfcinB)-derived peptide LfcinB₂₀₋₂₅ (RRWQWR), the low molecular weight LF hydrolysate (LFH<3 kDa), and two peptides identified in LFH<3 kDa (LIWKL and RPYL) were tested in *ex vivo* assays of vasoactive responses. The peptide RPYL was tested in radioligand receptor binding assays. Both LFH<3 kDa and individual peptides inhibited angiotensin II-induced vasoconstriction. RPYL showed the highest *ex vivo* inhibitory effect and also inhibited binding of [¹²⁵I]-(Sar¹, Ile⁸)-Angiotensin II to AT₁ receptors. By contrast, neither LFH<3kDa nor RPYL inhibited endothelin-1 and depolarization-induced vasoconstrictions. In conclusion, LF-derived peptides selectively inhibit angiotensin II-induced vasoconstriction by blocking angiotensin AT₁ receptors. Therefore, inhibition of angiotensin II-induced vasoconstriction is suggested as a mechanism contributing along with ACE inhibition to the antihypertensive effect of some LF-derived peptides.

KEYWORDS: *bovine lactoferrin hydrolysate, lactoferrin-derived peptides, antihypertensive mechanism, renin-angiotensin system, angiotensin-converting enzyme inhibition, angiotensin receptor blocker*

38 INTRODUCTION

39 Hypertension is probably the most important modifiable risk factor for cardiovascular
40 disease, and its management includes not only pharmacological treatment but also life-style
41 changes including physical activity and dietary habits.¹ The increasing perception about the
42 relation between food and health is fostering the development of functional foods providing
43 health benefits beyond basic nutrition.² Some food proteins contain embedded peptides that
44 once released behave as bioactive peptides with different health promoting properties
45 including blood pressure lowering effects.³

46 The renin angiotensin system (RAS) is one of the main targets for the treatment of
47 hypertension. Its inhibition at three possible levels, angiotensin-converting enzyme (ACE),
48 upstream renin activity or downstream angiotensin receptors, is the pharmacological basis for
49 commonly used antihypertensive drugs.⁴ ACE inhibition is also the most aimed target for
50 antihypertensive food-derived peptides developed as an alternative to drugs.⁵ Although
51 different animal and plant proteins have been used, milk is the main source of
52 antihypertensive ACE-inhibitory peptides reported to date.⁶

53 In contrast to casein, there was much less information about the potential of milk
54 whey proteins as sources of antihypertensive peptides when some years ago we focused on
55 whey protein lactoferrin (LF)⁷ and its well-known functional domain lactoferricin B (LfcinB).⁸
56 First, we have shown that a set of peptides derived from LfcinB had inhibitory effects on *in*
57 *vitro* ACE activity and *ex vivo* angiotensin I-induced ACE-dependent vasoconstriction,⁹ and
58 were orally effective antihypertensive peptides in spontaneously hypertensive rats (SHRs).¹⁰
59 With regard to the parent protein, we have reported the inhibitory effects of a LF pepsin
60 hydrolysate (LFH) on ACE activity and ACE-dependent vasoconstriction.¹¹ Then, we have
61 recently described that the ultrafiltered hydrolysate (LFH<3 kDa) showed increased inhibitory
62 effect on ACE activity and orally antihypertensive effect in SHRs after both acute¹² and
63 chronic¹³ administration. Finally, we have isolated and identified novel non-LfcinB-derived

peptides with ACE-inhibitory and antihypertensive properties, which could contribute to the antihypertensive effects of LFH<3 kDa.¹²

The search for *in vitro* ACE inhibition is the most common strategy in the selection of potential antihypertensive peptides derived from food proteins. However, it is difficult to establish a direct relationship between ACE-inhibitory effect *in vitro* and antihypertensive effect *in vivo*. On one hand, the *in vitro* ACE-inhibitory potency of food-derived peptides is around 1000 times lower than that of ACE-inhibitory drugs like captopril, but there are no substantial differences when their antihypertensive effects are compared.¹⁴ On the other hand, there is no correlation between *in vitro* and *in vivo* effects when different individual peptides or protein hydrolysates are compared. To explain these apparent discrepancies one should take into account both bioavailability issues and mechanisms of action other than ACE inhibition when bioactive peptides are assayed *in vivo*.¹⁵ Alternative antihypertensive mechanisms reported for food-derived bioactive peptides include short-term vasoactive mechanisms as well as long-term antioxidant and anti-inflammatory mechanisms.¹⁶

Looking for antihypertensive mechanisms beyond ACE inhibition in milk protein derived bioactive peptides, we have shown that some LfcinB-derived peptides were dual vasopeptidase inhibitors. In addition to ACE, they inhibit endothelin-converting enzyme (ECE), a key enzyme of the endothelin system also involved in vascular tone and blood pressure regulation.¹⁷ We have also reported that orally effective antihypertensive LF hydrolysates may act as dual vasopeptidase (ACE/ECE) or as single ECE inhibitors with different antivasoconstrictor effects depending on the protease used to release bioactive peptides.¹⁸ In the present study, we aimed to assess whether LF derived peptides, in addition to inhibit the converting enzymes of the angiotensin and endothelin systems, can act as receptor blockers to inhibit vasoconstriction induced by angiotensin II or endothelin-1. For this purpose, we included in the study the LfcinB-derived peptide LfcinB₂₀₋₂₅ (RRWQWR), the low molecular

weight LF pepsin hydrolysate LFH<3 kDa, and two peptides identified in LFH<3 kDa (LIWKL and RPYL), all of them with proven antihypertensive effects in SHR.

MATERIALS AND METHODS

Materials

Bovine lactoferrin (LF) was provided by FrieslandCampina Domo (Zwolle, The Netherlands). Porcine pepsin, porcine kidney ACE, human endothelin-1 (ET-1), captopril, valsartan and protease inhibitor cocktail were purchased from Sigma-Aldrich Química (Tres Cantos, Madrid, Spain). Angiotensin II was supplied by Calbiochem Co. (La Jolla, CA) and fluorescent ACE substrate o-aminobenzoyl-glycyl-p-nitrophenylalanylproline was from Bachem Feinchemikalien (Bubendorf, Switzerland). [¹²⁵I]-(Sar¹-Ile⁸)-Angiotensin II and angiotensin AT₁ receptor (human) membrane preparation were from PerkinElmer, Inc. (Waltham, MA), where membranes were prepared from transfected Chinese hamster ovary cells (cell line CHO-K1).

Peptides (LfcinB₂₀₋₂₅, LIWKL and RPYL) were ordered at >95% purity from GenScript Corp. (Piscataway, NJ) wherein they were synthesized by solid phase methods using N-(9-fluorenyl) methoxycarbonyl (Fmoc) chemistry. LfcinB₂₀₋₂₅ was acetylated at the N-terminus and amidated at the C-terminus, because such modified peptide was used in our previous studies showing its vasoactive [Centeno et al., 2006] and antihypertensive [Ruiz-Giménez et al., 2010] effects. Actual peptide purities of supplied batches were 99.8% for LfcinB₂₀₋₂₅, 97.7% for LIWKL and 99.5% for RPYL. Stock solutions of each peptide were prepared at 5 mM concentration in MilliQ water (Millipore Corp., Bedford, MA) and stored at −20°C. LfcinB₂₀₋₂₅ (RRWQWR) and LIWKL concentrations were determined on the basis of the extinction coefficient of W residues by measuring the absorbance at 280 nm ($\epsilon_{280} = 5600 \text{ M}^{-1} \text{ cm}^{-1}$ for W residue), while RPYL concentration was based on the dry weight of the peptide.

A low molecular weight fraction of an LF hydrolysate obtained by enzymatic treatment with pepsin (LFH<3 kDa) was prepared as described previously,^{11,12} and the resulting permeate was freeze-dried and kept at room temperature until use. LFH<3 kDa was subjected to determination of protein content by the bicinchoninic acid method (BCA; Sigma-Aldrich Química) using BSA as standard and to *in vitro* assay of ACE-inhibitory potency (IC₅₀) using the method described by Sentandreu and Toldrá¹⁹ as previously reported.¹²

Animal Welfare

Rabbits were housed in temperature-controlled rooms (23°C) with 12 h light/dark cycles and consumed tap water and standard diet *ad libitum*. Euthanasia was carried out in accordance with the Spanish legislation on 'Protection of Animals used for Experimental and other Scientific Purposes'. The study was approved by the 'Ethics Committee for Animal Welfare' of the Hospital 'La Fe' to be carried out in its accredited animal research facility (ES462500001001).

Isolation of Rabbit Carotid Arteries and Preparation of Membrane Microsomal Fraction

Thirty male New Zealand White rabbits (Technology Transferring Center, Polytechnic University of Valencia, Valencia, Spain), weighing 2.5–3 kg, were killed by injection of 25 mg/kg sodium thiopental and 1.5 ml of 10 mM KCl solution through the ear vein. A midline throat incision provided access to both common carotid arteries, which were dissected free and cut into four 4 mm-long segments each one for the *ex vivo* functional assays in organ bath. On the other hand, the membrane microsomal fraction from carotid arteries was obtained for radioligand binding assays according to Faber et al.²⁰ with some modifications. Briefly, frozen tissue was pulverized in liquid N₂, and homogenized (500 µL for 100 mg of tissue) in 4°C buffer (25 mM Tris-HCl, 1 mM EDTA, 2 mM MgCl₂, and 100 mM KCl, pH 7.4) plus protease inhibitor cocktail (1% v/v) with an Ultra-Turrax T8 homogenizer (IKA-Werke, Staufen Germany) at

maximal speed (5 x 10 s). All procedures specified below were carried out at 4°C. The homogenate was subjected to 3 strokes at 4 watts in a Microson XL ultrasonic cell disruptor (Misonix, Farmingdale, NY), treated with 0.6 M KCl for 20 min to remove major contractile proteins, centrifuged at 1,000g for 10 min, the supernatant centrifuged at 10,000g for 10 min, and again the supernatant ultracentrifuged at 110,000g for 1 h. The final pellet was resuspended in storage buffer (50 mM Tris-HCl, 0.5 mM EDTA, 10 mM MgCl₂, 10% sucrose, pH 7.4, protease inhibitor cocktail) and kept at -80°C until used. The concentration of membrane protein was determined by the bicinchoninic acid method.

***Ex Vivo* Functional Assays of Vasoactive Responses**

The arterial segments were mounted in an organ bath containing Ringer-Locke solution (120 mM NaCl, 5.4 mM KCl, 2.2 mM CaCl₂, 1.0 mM MgCl₂, 25 mM NaHCO₃, and 5.6 mM glucose) for computer-assisted isometric tension recording, as described previously.⁹ The contractile capacity of every arterial segment was checked by exposure to 50 mM KCl Ringer-Locke solution (NaCl replaced with an equimolar amount of KCl). Carotid arteries contracting less than 1 g were discarded.

To assess effects on angiotensin receptor-mediated vasoconstriction, every arterial segment was challenged with angiotensin II (1 µM). After washing out, each arterial segment was subjected to one of the following protocols: (i) control, a second challenge to angiotensin II to check for response reproducibility or (ii) treated, preincubation (20 min) with LFH<3 kDa (100 µg/mL), one of the LF-derived peptides (20 µM), the ACE-inhibitor drug captopril (0.1 µM) or the angiotensin II AT₁ receptor blocker drug valsartan (10 nM), and a second challenge to angiotensin II to check for their effect on angiotensin II-induced contraction. Concentration-dependent effects were assessed for the LF-derived peptide RPYL (20-200 µM) and valsartan (10-100 nM).

To assess effects on endothelin receptor-mediated vasoconstriction, every arterial segment was challenged with KCl (50 mM). After washing out, each arterial segment was subjected to one of the following protocols: (i) a challenge with endothelin-1 (10 nM) to obtain the control contractile response, or (ii) treated, preincubation (20 min) with LFH<3 kDa (100 µg/mL), the LF-derived peptide RPYL (20 µM) or the angiotensin II AT₁ receptor blocker drug valsartan (0.1 µM), and a challenge with endothelin-1 to check for their effect on endothelin-1-induced contraction.

Finally, some arterial segments were subjected to two successive challenges to 50 mM KCl with or without LFH<3 kDa (100 µg/mL), the LF-derived peptide RPYL (20 µM) or valsartan (0.1 µM) in between to check for non specific effects of the treatments on arterial contractile capacity.

Radioligand Receptor Binding Assays

Competition binding assays were carried out with both a membrane preparation containing human angiotensin AT₁ receptor and a membrane microsomal fraction from rabbit carotid artery. The human receptor membrane preparation (0.6 µg protein) was incubated at 27°C for 60 min in a total volume of 200 µL assay buffer (50 mM Tris-HCl, 5 mM MgCl₂, pH 7.4) with [¹²⁵I]-(Sar¹, Ile⁸)-Angiotensin II (0.03 nM) and various concentrations of unlabelled competitors. Experiments were terminated by rapid vacuum filtration through Whatman GF/C fiberglass filters (GE Healthcare Europe GmbH, Barcelona, Spain) presoaked in 0.5% BSA using a Brandel cell harvester (M24R, Valley Research Iberica, Madrid, Spain). The filters were then washed five times with 1 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4), and the filter-bound radioactivity was measured in a gamma counter (2480 WIZARD, PerkinElmer, Waltham, MA). Non-specific binding was determined in the presence of 1 µM unlabelled valsartan. Specific binding is defined as total binding minus non-specific binding. For the rabbit carotid artery membrane preparation, 40 µg protein and [¹²⁵I]-(Sar¹, Ile⁸)-Angiotensin II (0.06 nM) were used

instead. A full competition binding assay curve (1 pM - 1 μ M) was carried out for valsartan, and selected concentrations of RPYL were assayed according to the vasoactive responses in the *ex vivo* functional assays.

Statistical Analyses

Values are expressed as the mean \pm SEM. Unpaired Student's *t*-test was used to assess differences between two groups. Analysis of variance (ANOVA) followed by Student-Newman-Keuls test or Dunnett's test was used for multiple comparisons among more than two groups. *P*-values less than 0.05 were considered significant. The calculation of IC₅₀ values was performed by nonlinear regression analysis. GraphPad Prism software (GraphPad Software Inc., La Jolla, CA) was used.

RESULTS

Lactoferrin-Derived Peptides Inhibit Angiotensin II-Induced Vasoconstriction

Challenge of rabbit carotid arterial segments with a depolarizing solution (50 mM KCl) induced phasic contraction followed by active tone maintained until KCl wash-out. Average active tone amounted to 3424 ± 106 mg in all viable arterial segments included in the study (*n* = 200).

Angiotensin II (1 μ M) induced phasic, transient contractions that were almost completely reproducible in two consecutive challenges to the same arterial segment (Figure 1A). Average contractile response to the second challenge was 82 ± 2 % of the first response (*n* = 30). The inhibitory effects of the different treatments on angiotensin II-induced vasoconstriction were assessed as shown for RPYL in the representative recording of Figure 1B. LfcinB₂₀₋₂₅ (20 μ M), LFH<3 kDa (100 μ g/mL), LIWKL (20 μ M) and RPYL (20 μ M) produced significant inhibition of angiotensin II-induced vasoconstriction when compared to control vasoconstriction (Figure 2A). The degree of inhibition ranged from 21 % response reduction

over the control conditions for the weakest LfcinB₂₀₋₂₅ to 44 % response reduction for the strongest RPYL. Figure 2A also shows the lack of effect of the ACE-inhibitor captopril (0.1 μ M) on angiotensin II-induced vasoconstriction and the significant inhibitory effect of the angiotensin II AT₁ receptor blocker valsartan (10 nM). Finally, concentration-dependent inhibitory effects on the contractile response to angiotensin II were shown for RPYL (20-200 μ M) as well as for valsartan (10-100 nM) (Figure 2B).

Lactoferrin-Derived Peptide RPYL Inhibits Binding of [¹²⁵I]-(Sar¹, Ile⁸)-Angiotensin II to AT₁ Receptors

The competitive angiotensin II AT₁ receptor blocker valsartan (1 pM - 1 μ M) induced concentration-dependent inhibition of [¹²⁵I]-(Sar¹, Ile⁸)-Angiotensin II (0.03 nM) specific binding to human angiotensin AT₁ receptors (Figure 3A) with a calculated IC₅₀ value of 0.79 ± 0.15 nM (n = 6). The peptide RPYL (3-300 μ M) also induced significant concentration-dependent inhibition of [¹²⁵I]-(Sar¹, Ile⁸)-Angiotensin II specific binding to human angiotensin AT₁ receptors (Figure 3B), amounting to 62 % binding inhibition at the highest RPYL concentration assayed (300 μ M). On the other hand, RPYL (100 μ M) also significantly reduced to 38.2 ± 5.0 % (n = 3, *P* < 0.01) specific binding of [¹²⁵I]-(Sar¹, Ile⁸)-Angiotensin II (0.06 nM) to rabbit carotid artery angiotensin AT₁ receptors.

Lactoferrin-Derived Peptides Do Not Inhibit Endothelin-1 and Depolarization-Induced Vasoconstrictions

Endothelin-1 (10 nM) induced slow contraction (Figure 4A) with maximum effect amounting to 108 ± 5 % relative to previous response to KCl, reached at 67 ± 6 min, with mean slope of 61 ± 7 mg/min (n = 16). The effects of the different treatments on endothelin-1-induced vasoconstriction were assessed as shown for LFH<3 kDa in the representative

recording of Figure 4B. LFH<3 kDa (100 µg/mL), the peptide RPYL (20 µM) or valsartan (0.1 µM) did not induce significant modification of the contractile response to endothelin-1 (Table 1).

Finally, the effects of some treatments inhibiting angiotensin II-induced vasoconstriction were assayed also against KCl-induced vasoconstriction. In control conditions, the second vasoconstriction to KCl-induced depolarization was 116 ± 2 % of the first response in the same arterial segment ($n = 19$), and the responses to KCl were not significantly different after incubation with LFH<3 kDa (100 µg/mL), RPYL (20 µM) or valsartan (0.1 µM) (Table 1).

DISCUSSION

We have found that some lactoferrin (LF)-derived antihypertensive peptides inhibit angiotensin II-induced vasoconstriction due to their blocking effect on angiotensin AT₁ receptors. This effect is selective for angiotensin AT₁ receptors as endothelin-1-induced, ET_A receptor-mediated vasoconstriction is not inhibited, and no unspecific effects on depolarization-induced vasoconstriction are shown.

We have previously reported that the low molecular weight LF hydrolysate included in this study (LFH<3 kDa) shows antihypertensive effects in SHR. Single-dose LFH<3 kDa induces acute blood pressure reduction in SHR,¹² and long-term intake attenuates progression of hypertension.¹³ LFH<3 kDa inhibits both *in vitro* ACE activity¹² and ACE-dependent angiotensin I-induced vasoconstriction.¹⁸ The ACE inhibitory effect of LFH<3 kDa has been confirmed as an *in vivo* mechanism for the antihypertensive effect.¹³ The present results point to inhibition of angiotensin II-induced vasoconstriction as a potential mechanism also contributing along with ACE inhibition to the antihypertensive effect of the hydrolysate. We have also previously shown that individual peptides embedded in different regions of the LF sequence, either derived from the lactoferricin B domain (LfcinB) or identified in LFH<3 kDa, are ACE inhibitors and have antihypertensive effects when acutely administered to SHR.^{9,10,12} The present study shows that LfcinB₂₀₋₂₅ also has a slight inhibitory effect on angiotensin II-induced

vasoconstriction. On the other hand, the inhibitory effects of individual peptides like LIWKL and to a higher extent RPYL on angiotensin II-induced vasoconstriction would contribute at least in part to the inhibitory effect of the whole hydrolysate LFH<3 kDa.

The mechanism involved in the inhibitory effects of LF-derived peptides on angiotensin II-induced vasoconstriction has been further investigated using the most effective peptide RPYL. On one hand, our results show that the inhibitory effect of RPYL on angiotensin II-induced vasoconstriction was concentration-dependent. Moreover, RPYL displaced the binding of [¹²⁵I]-(Sar¹, Ile⁸)-Angiotensin II to human angiotensin AT₁ receptors also in a concentration-dependent manner. These human recombinant receptors, expressed in transfected Chinese hamster ovary cells, are commonly used for AT₁ receptor binding assays both in whole cells [Verheijen et al., 2000] and membrane preparations [Lee et al 2013]. Finally, the inhibitory effect of RPYL on [¹²⁵I]-(Sar¹, Ile⁸)-Angiotensin II specific binding was confirmed in membranes from rabbit carotid artery, the native tissue used in vasoconstriction experiments. All these results support an angiotensin AT₁ receptor-blocking mechanism for the inhibitory effect of RPYL on angiotensin II-induced vasoconstriction. Angiotensin receptor blocker (ARB) drugs are a series of chemically related imidazol derivatives used in the clinical setting for hypertension treatment because of their ability to bind to angiotensin AT₁ receptors, thereby inhibiting vasoconstriction and other cellular actions of angiotensin II.^{21,22} Valsartan, a member of this drug family²³ has been used in our study as positive control both in vasoconstriction and binding experiments. Taking into account the concentrations used, RPYL was much less potent than valsartan in both experimental settings.

The inhibitory effects of food-derived antihypertensive peptides on angiotensin II-induced vasoconstriction have not been extensively studied, and when done no inhibition has been found. Others have reported the lack of effect of the milk-derived tripeptide IPP²⁴ and the sardine muscle-derived dipeptide VY²⁵ on angiotensin II-induced vasoconstriction. In a previous study with rationally designed heptapeptides, we found that the ACE inhibitor

antihypertensive heptapeptide PACEI50 (RKWHFLW) did not modify angiotensin II-induced vasoconstriction.²⁶ With regard to LF-derived peptides, the crude LF pepsin hydrolysate before ultrafiltration (LFH) did not inhibit angiotensin II-induced vasoconstriction, did not modify blood pressure in SHR, and showed much less inhibitory potency on ACE than LFH<3 kDa,^{11,12} thus indicating that the antihypertensive effects of the LF hydrolysate are due to the bioactive properties of its low molecular weight peptides like LIWKL and RPYL.

To the best of our knowledge, the present study for the first time shows the ability of antihypertensive bioactive peptides to inhibit angiotensin II-induced vasoconstriction by an angiotensin AT₁ receptor blocking mechanism. With regard to non peptide substances, inhibition of angiotensin II-induced vasoconstriction has been reported to underlie the antihypertensive effects of compounds like nitro-oleic acid, a NO-derived fatty acid,²⁷ and especially plant extracts used in folk medicine.²⁸⁻³² Moreover, proanthocyanidins and alkaloids from some plant extracts are angiotensin AT₁ receptor binding compounds.³³⁻³⁵ On the other hand, inhibitory effects on renin activity, the rate limiting enzyme upstream the RAS,³⁶ have been reported for hemp seed protein hydrolysates,³⁷ flaxseed protein hydrolysate fractions³⁸ and peptides from pea protein hydrolysates,³⁹ as well as for peptides from a protein hydrolysate of the macroalga *Palmaria palmate*.⁴⁰ In most of these cases, inhibitory effects were observed on both renin and ACE activities.³⁷⁻³⁹ Therefore, bioactive peptides with antihypertensive potential can be multifunctional peptides inhibiting more than one of the RAS steps: generation of angiotensin I from angiotensinogen by renin, generation of vasoactive angiotensin II from angiotensin I by ACE, or activation of the AT₁ receptor by angiotensin II.³⁶

The endothelin system, similar to the RAS, is another peptidic system with an increasingly recognized role in blood pressure regulation and hypertension pathophysiology.⁴¹ Of particular relevance in the cardiovascular system, endothelin-1 has powerful vasoconstrictor and pressor properties. The gene product preproendothelin-1 is cleaved by a furin-like endopeptidase to form a biologically inactive intermediate termed big endothelin-1.

Next, big ET-1 is cleaved by an endothelin-converting enzyme (ECE) to form endothelin-1, which binds to vascular ET_A receptors to induce vasoconstriction.⁴² Both AT₁ and ET_A receptors belong to the class 1 of G protein-coupled receptors,⁴³ and dual action AT₁ and ET_A receptor antagonists reduce blood pressure in experimental hypertension.⁴⁴ Moreover, angiotensin AT₁ receptor binding alkaloids from the root of *Bocconia frutescens* also show weaker binding to endothelin ET_A receptors.³⁴ Our results show that neither LFH<3 kDa nor RPYL, the peptide with the highest inhibitory effect on angiotensin II-induced vasoconstriction, modified the vasoconstriction induced by endothelin-1, thus supporting the selective effects of LF-derived peptides on the AT₁ receptor. In a previous study, we reported that LfcinB-derived peptides hinder endothelin-1 generation by inhibiting ECE activity but do not act on downstream ET_A receptors or intracellular signal transduction mechanisms leading to vasoconstriction.¹⁷

Some considerations have to be made regarding availability of LF-derived peptides both in the present experiments and in *in vivo* circumstances. On one hand, peptidases are present in the wall of blood vessels, both in the endothelial layer and in the smooth muscle.^[Palmieri et al., 1989] In addition to ACE^[Centeno et al., 2006] and ECE^[Fernández-Musoles et al., 2010] activities previously shown in the isolated rabbit carotid artery used in our *ex vivo* assays, some other vascular peptidases hydrolyze endogenous vasoactive peptides thus interfering with their biological effects.^[Ryan, 1989; Rouissi et al., 1990] A possible hydrolytic effect of vascular peptidases on LF-derived peptides during their incubation with the carotid arteries cannot be dismissed. On the other hand, depending on the acid base nature of their amino acid residues, peptide-peptide ionic interactions can occur to form quite stable non covalent complexes.^[Woods & Huestis, 2001] In our study, interaction between LF-derived peptides and angiotensin II to form peptide complexes unable to bind to AT₁ receptors has not been assessed. The influence of such mechanism on availability of LF-derived peptides and their inhibitory effects on angiotensin II-induced contraction cannot be discarded. Finally, bioavailability of peptides after oral administration is needed to guarantee bioactivity.^[14] The

few bioavailability studies performed *in vivo* show the detection of VPP and IPP in plasma in picomolar to nanomolar concentrations[Foltz et al., 2007] with elimination half-lives of about 2 min[Van der Pijl et al., 2008]. The low bioavailability found for most natural peptides raises questions as to whether those peptides lower blood pressure only via an ACE-inhibiting mechanism in blood vessels. Although plasma levels of the peptides were not measured in our studies reporting the antihypertensive effects of orally administered LFc_{in} B₂₀₋₂₅, LIWKL and RPYL,[Ruiz-Giménez et al., 2010; 2012] we showed RPYL to be particularly resistant to simulated gastrointestinal digestion[Ruiz-Giménez et al., 2012]. Moreover, RPYL and the other LF-derived peptides add their inhibitory effects on angiotensin II effects to ACE inhibition.

In conclusion, LF-derived peptides released from different regions of the protein inhibit angiotensin II-induced vasoconstriction by blocking angiotensin AT₁ receptors. The lack of effects on endothelin-1-induced, ET_A receptor-mediated vasoconstriction as well as on depolarization-induced vasoconstriction points to a selective effect of the peptides. Therefore, inhibition of angiotensin II-induced vasoconstriction is suggested as a potential mechanism also contributing along with ACE inhibition to the antihypertensive effect of some LF-derived peptides.

361 **ABBREVIATIONS USED**

362 ACE, angiotensin-converting enzyme; AT₁, angiotensin receptor 1; ECE, endothelin-converting
363 enzyme; ET-1, endothelin-1; ET_A, endothelin receptor A; IC₅₀, half-maximal effective
364 concentration; LF, lactoferrin; LfcinB, lactoferricin B; LFH, lactoferrin hydrolysate; RAS, renin
365 angiotensin system; SHR, spontaneously hypertensive rat

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FIGURE CAPTIONS

Figure 1. Typical vasoconstrictor effects of angiotensin II (Ang II) in carotid artery segments isolated from New Zealand White rabbits. (A) Successive vasoconstrictions to Ang II in control conditions. (B) Effect of the LF-derived peptide RPYL on Ang II-induced vasoconstriction.

Figure 2. Inhibitory effects of lactoferrin-derived peptides on angiotensin II (Ang II)-induced vasoconstriction in carotid artery segments isolated from New Zealand White rabbits. (A) LfcinB₂₀₋₂₅, LFH<3 kDa, the LF-derived peptides LIWKL and RPYL, the ACE-inhibitor captopril, and the Ang II AT₁ receptor blocker valsartan were applied before the second addition of Ang II (1 μ M). (B) Concentration-dependent inhibitory effects of RPYL and valsartan on Ang II-induced vasoconstriction. Vasoconstrictor responses are expressed as a percentage of previous Ang II-induced contraction. Data are the mean \pm SEM from (n) arterial segments. * P < 0.05 and ** P < 0.01 compared with Ang II alone (control), respectively. ## P < 0.01 compared with RPYL (20 μ M), and § P < 0.05 compared with valsartan (10 nM).

Figure 3. Inhibition of [¹²⁵I]-(Sar¹,Ile⁸)-Angiotensin II specific binding to a membrane preparation from CHO-K1 cells expressing the human angiotensin AT₁ receptor. Non-specific binding was 29.4 \pm 4.1%, mean \pm SEM from nine independent experiments (each performed in duplicate or triplicate). (A) Sigmoidal curve for concentration-dependent inhibitory effects of the angiotensin II AT₁ receptor blocker valsartan. Data are the mean \pm SEM from six independent experiments (each performed in duplicate or triplicate). (B) Concentration-dependent inhibitory effects of the LF-derived peptide RPYL. Data are the mean \pm SEM from at least three independent experiments (each performed in duplicate or triplicate). * P < 0.05 and ** P < 0.01 compared with [¹²⁵I]-(Sar¹,Ile⁸)-Angiotensin II alone (control), respectively. # P < 0.05 compared with RPYL (30 μ M).

546 **Figure 4.** Typical vasoconstrictor effects of endothelin-1 (ET-1) in carotid artery segments
547 isolated from New Zealand White rabbits. (A) Vasoconstriction to high-KCl and to ET-1 in
548 control conditions. (B) Effect of LFH<3 kDa on ET-1-induced vasoconstriction.

Table 1. Vasoconstrictor responses of carotid artery segments isolated from New Zealand White rabbits to endothelin-1 and KCl-induced depolarization, in control conditions and after treatment with LFH<3 kDa, the LF-derived peptide RPYL, the angiotensin II AT₁ receptor blocker valsartan, and the selective ET_A endothelin receptor antagonist BQ-123 .

	Endothelin-1 (10 nM)	KCl (50 mM)
Control	124 ± 2 (3)	117 ± 2 (8)
LFH<3 kDa (100 µg/mL)	113 ± 6 (9)	113 ± 2 (8)
Control	106 ± 11 (7)	109 ± 2 (7)
RPYL (20 µM)	93 ± 5 (8)	109 ± 3 (7)
Control	103 ± 3 (6)	124 ± 8 (4)
Valsartan (0.1 µM)	109 ± 3 (16)	128 ± 5 (12)
Control	123 ± 7 (12)	n.d.
BQ-123 (1 µM) ^a	49 ± 3 (8)**	

Vasoconstrictor responses are expressed as a percentage of previous KCl-induced contraction.

Data are the mean ± SEM from (n) arterial segments (n.d., not determined).

^aTaken from Fernández-Musoles et al.¹⁷

** Significantly different from control, $P < 0.01$. Unpaired Student's *t*-test.